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PRINCIPAL INVESTIGATOR: T. Keith Blackwell, M.D., Ph.D.

CONTRACTING ORGANIZATION: Center for Blood Research
Boston, Massachusetts 02115

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T. Keith Blackwell, M.D., Ph.D.

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Center for Blood Research
Boston, Massachusetts 02115

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Abnormalities in c-*myc* regulation are associated with breast carcinoma. We have begun to investigate molecular interactions that are functionally essential for c-Myc and other basic-helix-loop-helix (bHLH) proteins, both to illuminate aspects of their biology and to provide a basis for future design of therapeutics. To study bHLH dimerization, we are continuing to construct and analyze mutants in the bHLH protein MyoD. We have also expanded our investigations of how different bHLH proteins recognize different related DNA sequences. In these experiments, we are focusing on the role of basic segment residues that have been implicated in transcriptional activation, and are testing the prediction that these residues are critical for proper positioning of the DNA-bound basic regions. We have also begun a new set of experiments to investigate whether the Mastermind protein, which contains a single basic region, is a DNA-binding protein. Mastermind has been implicated as a critical element in the functioning of Notch proteins, which in turn have been implicated in mammary oncogenesis.

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No animal research was performed TKB

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INTRODUCTION:

c-Myc and breast cancer:

Amplification of the c-*myc* gene has been reported in as many as 20-30% of sporadic breast tumors, and may be associated with a relatively poor prognosis (Escot et al., 1986; Watson et al., 1993). Of perhaps even greater significance is the observation that constitutive expression of c-Myc predisposes mammary tissue to carcinoma (Schoenenberger et al., 1988; Stewart et al., 1984), as is the case in many other cell lineages (Leder et al., 1986). The potential implications of uncontrolled c-Myc expression are further illustrated by the finding that it can allow cells to become transformed without an accompanying mutation in the tumor suppressor gene p53 (Lu et al., 1992), abnormalities of which are associated with both sporadic and hereditary breast cancer (Harris et al., 1992). An investigation of c-Myc function is therefore relevant to breast cancer not only because of its specific association with mammary carcinoma, but also because activation of the cellular regulatory networks in which it is involved seems in general to contribute to oncogenesis. These observations suggest that c-Myc will be a promising target for development of future antineoplastic therapies which are designed specifically to inhibit its function.

To generate such molecularly-based therapies, it will be important not only to identify cellular factors that c-Myc activates (and is activated by), but also to understand the specific molecular interactions involved. In this project, we have begun to address this last issue. Using structural information as a guide, we are identifying particular protein-protein and protein-DNA interactions that are essential for the function and target specificity of helix-loop-helix proteins, including c-Myc. A detailed understanding of these intermolecular interactions is essential for an understanding of c-Myc biology and necessary for design of therapeutics.

c-Myc as a transcriptional regulator:

Evidence suggests that c-Myc is involved in regulating progression through the cell cycle (Jansen-Durr et al., 1993; Luscher and Eisenman, 1990). In the mouse, both the c- and N-*Myc* genes are essential for development, but either can be disrupted without impairing the viability of individual embryonic stem cells (Charron et al., 1992; Davis et al., 1993; Moens et al., 1992; Sawai et al., 1993; Stanton et al., 1992). These latter findings demonstrate that Myc proteins are not parts of the essential cell cycle machinery, and suggest instead that they transmit proliferative signals to it. Indeed, c-Myc seems to interact with multiple cellular signalling pathways, as is indicated by the apparent complexity of its transformation-inducing capability (Lu et al., 1992; Sawyers et al., 1992), and by the observation that in cells which have been deprived of growth factors, expression of c-Myc can induce apoptosis (Evan et al., 1992; Neiman et al., 1991). Apparently, c-Myc is part of a regulatory network that induces apoptosis if the cell is receiving mixed or inappropriate signals regarding whether to proliferate (Shi et al., 1992). Thus, while over-expression of c-Myc appears to contribute to cellular transformation by inducing proliferation (Luscher and Eisenman, 1990), the pathways by which it does so seem to be complex.

An essential insight into how c-Myc might perform these functions has come from the realization that Myc proteins are members of the basic-helix-loop-helix (bHLH) family of DNA-binding proteins (Figure 1) (Davis et al., 1987; Murre et al., 1989). In general, members of this large family are involved in transcriptional regulation, with some playing a role in cellular differentiation, and others implicated in oncogenesis (Weintraub et al., 1991). They are defined by the HLH domain (Murre et al., 1989; Murre et al., 1989), which allows them to form dimers, and by a region of

basic amino acids (BR) which lies immediately N-terminal to this domain and through which they bind to specific DNA sequences (Davis et al., 1990; Voronova and Baltimore, 1990). Myc proteins belong to a bHLH subgroup (bHLH-ZIP proteins) in which a "leucine zipper" (ZIP) domain (Landschulz et al., 1988) is located immediately C-terminal to the HLH domain (Blackwood and Eisenman, 1991), and provides a critical contribution to dimerization (Beckmann and Kadesch, 1991; Davis and Halazonetis, 1993; Ferre-D' Amare et al., 1993; Fisher et al., 1991; Halazonetis and Kandil, 1992; Ma et al., 1993). ZIP domains form an amphipathic α-helix that dimerizes as a coiled-coil (O'Shea et al., 1989), and thus also define a separate family of transcriptional regulatory proteins (the b-ZIP proteins) (Johnson and McKnight, 1989). bHLH proteins must form dimers to bind to DNA (Davis et al., 1990; Voronova and Baltimore, 1990), and generally recognize sites that contain the palindromic consensus CA -- TG (Lassar et al., 1989), with each respective BR binding to half of the site (Blackwell and Weintraub, 1990; Ferre-D' Amare et al., 1993). Some bHLH protein family members readily form homodimers, but others do not, and appear to require a different dimerization partner (Weintraub et al., 1991). For example, while Myc protein bHLH-ZIP domains can bind DNA <u>in vitro</u> as homodimers (Alex et al., 1992; Blackwell et al., 1990; Kerkhoff et al., 1991; Ma et al., 1993), they dimerize (and thus bind DNA) far more efficiently as heterodimers with Max, a widely-expressed bHLH-ZIP protein (Blackwood and Eisenman, 1991; Prendergast et al., 1991) which is required for their capacity to transform cells, and appears to be essential for their normal functions (Amati et al., 1993; Blackwood et al., 1992; Kato et al., 1992; Mukherjee et al., 1992; Prendergast et al., 1992; Wenzel et al., 1991).

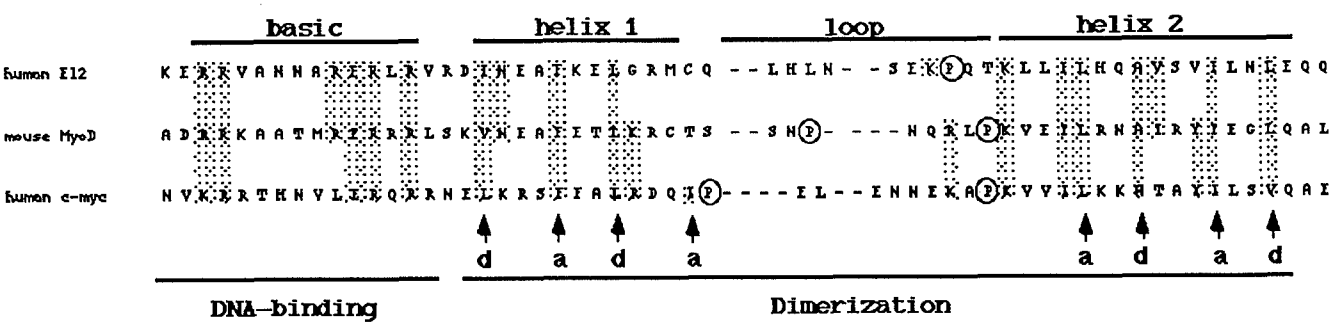


Figure 1:
Representative bHLH domains (taken from Benezra, et al., 1990). Conserved amino acids are shaded. Positions that correspond to the a and d positions of amphipathic alpha helices are indicated. Helix-disrupting proline residues are circled. MyoD BR residues are numbered in the text so that the left-most shaded R residue corresponds to 1.

By analogy to other bHLH proteins, it would be predicted that Myc proteins would be involved in transcriptional regulation (Collum and Alt, 1990; Luscher and Eisenman, 1990). Recent experiments support this idea (Amati et al., 1992; Amin et al., 1993; Gu et al., 1993; Kato et al., 1990; Kretzner et al., 1992), and have suggested the following model: c-Myc/Max and Max/Max complexes compete for the same DNA targets, at which c-Myc/Max activates and Max/Max blocks transcription (Amati et al., 1992; Kretzner et al., 1992). Direct repression can be achieved at these sites by binding of heterodimers of Max with the bHLH-ZIP proteins Mad (Ayer et al., 1993) or Mxi (Zervos et al., 1993). In the remainder of this report, this group of proteins will be referred to as the Myc/Max/Mad/Mxi network, because they appear to be linked in function by their abilities to interact with each other and to recognize common DNA sequences. In addition to its apparent function as an activator, c-Myc can inhibit

transcriptional initiation which is mediated by the TFII-I protein, a finding which may explain how c-Myc appears to repress transcription of particular genes (Roy et al., 1993). In recent years, the list of putative direct or indirect targets of the Myc/Max/Mad/Mxi network has been growing longer, and includes proteins associated with cell division, such as cyclins and regulators of cyclin-dependent kinase activity (Bello-Fernandez et al., 1993; Benvenisty et al., 1992; Born et al., 1994; Daksis et al., 1994; Eilers et al., 1991; Galaktionov et al., 1996; Grandori et al., 1996; Hann et al., 1994; Jansen-Durr et al., 1993; Jones et al., 1996).

The two c-Myc regions which are indispensible for its transforming capability (Stone et al., 1987) were later identified as its transcriptional activator and bHLH-ZIP domains, indicating the importance of its ability to regulate transcription. Myc proteins are remarkably conserved in these regions (Schreiber-Agus et al., 1993; Walker et al., 1992), and can all bind to sites that contain CACGTG or CATGTG core sequences (Alex et al., 1992; Berberich et al., 1992; Blackwell et al., 1990; Kato et al., 1992; Ma et al., 1993; Papoulas et al., 1992), suggesting that they may have similar or overlapping functions. However, a number of related bHLH proteins, including the bHLH-ZIP transcriptional regulatory proteins USF, TFE3, and TFEB, can also bind to the same sequences (Beckmann et al., 1990; Carr and Sharp, 1990; Gregor et al., 1990). All of these bHLH-ZIP proteins contain in their respective BRs an arginine (R) residue (R_{13}) which is essential for recognition of these particular CA -- TG sites (Blackwell et al., 1993; Dang et al., 1992; Halazonetis and Kandil, 1992; Van Antwerp et al., 1992), and which directly contacts the central bases in them (Ferre-D' Amare et al., 1993). These similarities in DNA recognition raise the issue of how Myc proteins and these other bHLH-ZIP proteins might be able to act on different genes, and would appear to suggest that any differences in their target specificities would necessarily be determined by interactions with cooperating factors. Conversely, some differences in DNA recognition have been identified among them (Blackwell et al., 1993; Fisher and Goding, 1992; Halazonetis and Kandil, 1991; Prochownik and Van Antwerp, 1993). For example, the ability to bind to certain "non-canonical" sites, which are based on variants of the CA -- TG consensus, is shared by the Myc/Max/Mad proteins, but not by the other related bHLH-ZIP proteins, indicating that it might confer some degree of specificity and thus be of biological significance (Blackwell et al., 1993). Such DNA sequences have been found recently to be associated with a number of candidate Myc-responsive genes (Grandori et al., 1996).

The relationship between DNA-binding and transcriptional regulation by c-Myc may be complex, as is suggested by the example of the bHLH protein MyoD (Figure 1). MyoD induces many cell types to differentiate into muscle (Davis et al., 1987; Weintraub et al., 1989), and it functions as a heterodimer with members of the widely-expressed E2A family of bHLH proteins (i. e. E12; Figure 1) (Lassar et al., 1991; Murre et al., 1989). Mutational analyses of MyoD and of related bHLH proteins have shown that certain BR mutations allow them to bind to appropriate DNA sequences but interfere with their ability to activate transcription or induce myogenesis (Davis et al., 1990; Davis and Weintraub, 1992; Schwarz et al., 1992; Weintraub et al., 1991). These findings suggest that the MyoD BR is involved in protein-protein interactions as well as in binding to DNA (Weintraub et al., 1991). Such a mechanism (referred to as "positive control"; (Hochschild et al., 1983)) has been described in other families of DNA-binding proteins (Kristie and Sharp, 1990; Lai et al., 1992; Stern et al., 1989). In the case of MyoD, it has been proposed that appropriate protein-DNA and protein-protein interactions are required for exposure of its transcriptional activator domain, which appears to be "buried" within the protein when its BR is not bound to DNA (Weintraub et al., 1991). This mechanism could potentially contribute to target

specificity, if only a subset of MyoD binding sites were to allow binding in a conformation which would permit these protein-protein interactions to occur, and thus were capable of inducing transcriptional activation (Weintraub et al., 1991). Such complex mechanisms for determining target specificity could potentially be utilized by other bHLH proteins, including those of the Myc family. In fact, members of different bHLH protein groups, including the Myc proteins, are characterized by particular amino acids in their BRs for some of which no direct role in determining DNA-binding specificity has yet been demonstrated (Ferre-D' Amare et al., 1993; Fisher et al., 1993). The conservation of these amino acids suggests biological importance, either for as yet undetermined effects on DNA-binding, for protein-protein interactions, or both.

bHLH protein structure:

Recent insights into c-Myc protein-protein and protein-DNA interactions present the prospect that in the future, antineoplastic therapeutics might be designed to interfere with them (see (Perutz, 1992)), and thus block the ability of c-Myc to regulate transcription. The determination of structures for bHLH protein-DNA complexes represents a major step forward in this direction. These efforts have shown that Max forms a parallel, left-handed, four helix bundle in which the ZIP domain continues C-terminally from helix 2, and the BR extends as an α-helix N-terminally from helix 1 as it crosses the major groove of B-form DNA (Ferre-D' Amare et al., 1993). Recently-determined structures for complexes of the bHLH proteins E47 and MyoD (which lack a ZIP domain) bound to DNA has further revealed that the configuration of the HLH domain fold is remarkably preserved between bHLH and bHLH-ZIP proteins (Ellenberger et al., 1994; Ma et al., 1994). While these structures have demonstrated how the HLH dimerization interface is formed, and have made predictions about critical protein-DNA contacts which can now be tested, they also leave open a number of questions. For example, they have not suggested roles for a number of BR residues which do not contact bases, yet are conserved within different bHLH protein sub-families (Benezra et al., 1990), and thus might be essential for their function. It is also not clear why bHLH-ZIP proteins require the ZIP domain for dimerization, or what determines the dimerization specificities of HLH domains. In addition, because these structures were determined using isolated bHLH or bHLH-ZIP domains, they do not address potential interactions between them and the remainder of these proteins.

Significantly, these studies do provide an essential basis for investigating such issues by a program of integrated mutagenesis, biochemical, and molecular modeling experiments. As a part of this research effort I have undertaken such an effort in collaboration with Dr. Thomas Ellenberger, who is investigating bHLH protein structure by X-ray crystallography and will incorporate our findings into further structural investigations. Our goal is to gain insights into the specificity of these protein-DNA and protein-protein interactions that will contribute to our understanding of the biology of c-Myc and of other bHLH proteins, and that will thus be an essential complement to efforts underway in other laboratories to identify Myc-responsive genes. Results from our experiments should thus be of particular value for future efforts at "rational" molecular design of antineoplastic therapies.

The Mastermind protein, Notch signaling, and mammary oncogenesis:

In a change of specific aim which has been approved by the Army, our second aim is now a study of DNA binding by the Mastermind protein.

Like bHLH proteins, other BR-containing proteins generally bind to DNA as dimers. For example, the bZIP proteins bind to DNA only as dimers, through a distinct type of BR which also lies in the major groove (Ellenberger et al., 1992). However,

one example of monomeric BR-DNA binding has been identified, the SKN-1 protein of C. elegans (Blackwell et al., 1994; Bowerman et al., 1992). SKN-1 contains at its C-terminus a BR like that of bZIP proteins, but it lacks a dimerization domain. Instead, it binds to DNA sequence-specifically as a monomer, by means of an 85 residue domain that places a flexible N-terminal "arm" into the minor groove of an AT-rich region, and stabilizes the BR by means of a predominantly helical intervening region (Blackwell et al., 1994). Only one other example has been identified of a BR that lacks a ZIP or an HLH segment, the Mastermind (Mam) protein of Drosophila (Smoller et al., 1990). Mam contains a BR (Figure 2), but lacks sequences that are similar to either SKN-1 or bZIP proteins.

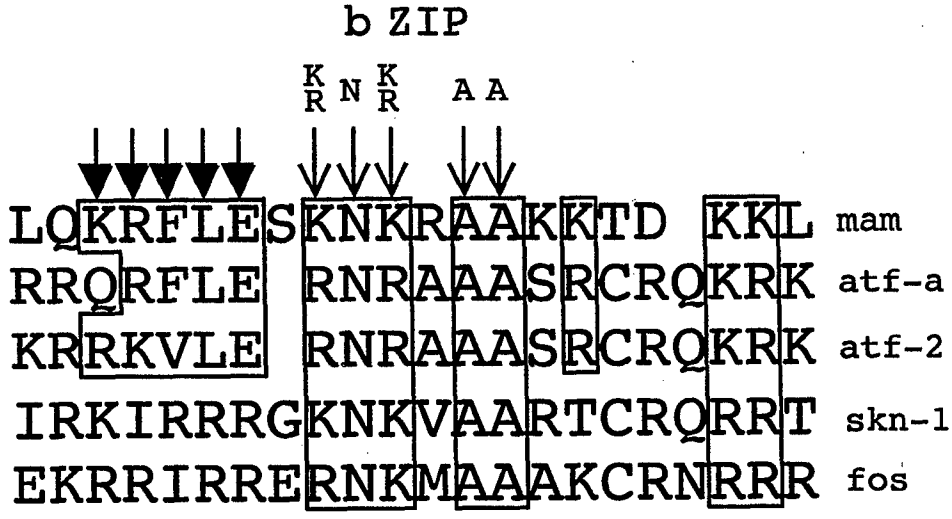


Figure 2:
Alignment of the Mastermind BR with representative BRs (Smoller et al., 1990). Conserved residues are boxed, and indicated with arrows.

Mam is relevant to breast cancer because it is required for implementation of signaling in response to Notch proteins (Artavanis-Tsakonas et al., 1995; Smoller et al., 1990), which have been implicated in mammary oncogenesis (see below). Notch is a transmembrane protein which is involved in numerous embryonic signaling events, in which cells are directed to follow or to suppress programs of differentiation. Proteins related to Notch have been found in organisms as diverse as C. elegans and humans, and are utilized in myriad decisions of cell fate in the developing embryo (Artavanis-Tsakonas et al., 1995). At least in part, in both Drosophila and vertebrates, the Notch signal appears to be effected through transcriptional activation by the Suppressor of Hairless (Su(H)) protein, which binds to regulatory sequences at target genes (Fortini and Artavanis-Tsakonas, 1994). Expression of a truncated Notch protein, which lacks the extracellular domain, results in a constitutive Notch signal (Artavanis-Tsakonas et al., 1995). Significantly, this Notch fragment has been demonstrated to associate with DNA-bound Su(H), and thus to convert it to an activator (Jarriault et al., 1995). It has been proposed that transduction of the Notch signal involves proteolytic cleavage which liberates this Notch fragment, and allows it to be translocated to the nucleus (Jarriault et al., 1995). As Mam is a nuclear protein, it is likely to be involved in these activation events, or in the functioning of gene products that are expressed in response to Notch signaling.

Insertion of the mouse mammary tumor virus (MMTV) into the mouse *int*-3 gene, a Notch family member, results in generation of mammary cell tumors (Robbins et al., 1992; van Leeuwen and Nusse, 1995). This transformation event appears to be

mediated by production of a constitutively-active truncated Int-3 protein, and expression of such a protein in mammary cells interferes with their differentiation and results in their transformation (Jhappan et al., 1992; Smith et al., 1995). These findings implicate activation of the Notch pathway in mammary carcinoma. Analogous activated Notch proteins have been linked to lymphoid tumors (Artavanis-Tsakonas et al., 1995), and have been demonstrated to cooperate with c-Myc to induce thymomas (Girard et al., 1996). An understanding of this pathway is therefore relevant to breast cancer, and to cancer in general.

We will attempt to identify specific DNA sequences that are bound by the Drosophila Mastermind protein, either alone, or together with candidate cooperating co-factors such as Su(H). These experiments will consist of *in vitro* selections from random sequence libraries (Blackwell, 1995), as well as gel mobility shift assays using sequences from candidate target genes. As these experiments yield results, we will move on to investigation of how the newly-described Mastermind DNA-binding domain might recognize DNA (and associated proteins), and perform cell culture investigations of Mastermind function. These experiments will also serve as a basis for future attempts to identify vertebrate mastermind genes.

BODY:

A. Investigation of c-Myc and bHLH protein-protein and protein-DNA interactions.

1. Protein-protein interactions:

The dimerization specificities of ZIP domains appear to be determined by interactions between charged amino acids which lie adjacent to their dimerization interface (O'Shea et al., 1992; Vinson et al., 1993), and evidence suggests that interactions between the ZIP domains of c-Myc and Max follow similar principles (Amati et al., 1993). However, it is not understood how the dimerization specificities of HLH domains are determined, nor is it known why the HLH domains of bHLH-ZIP proteins such as c-Myc and Max do not dimerize efficiently, so that they generally require the ZIP domain (see above). To address these issues, we have begun to use bHLH protein structures that were derived by X-ray crystallography as a starting point for mutational analyses and molecular modeling experiments.

Although in bHLH-ZIP proteins the HLH domain is not sufficient to mediate dimerization, its integrity appears to be required for dimer formation (Davis and Halazonetis, 1993; Reddy et al., 1992), and it is critical for orientation of the BRs (Ferre-D' Amare et al., 1993). The HLH domain does not follow the paradigm represented by the ZIP domain, in which hydrophobic residues that are present at positions a and d in the helix form a dimerization interface, with the remaining residues generally being polar (see (Ellenberger et al., 1992); Figure 1). Instead, in the HLH domain many of the residues at the g and e positions are hydrophobic, especially in helix 2, and the dimerization interface is in fact a core between the four helices, which is shielded from solvent exposure (Ferre-D' Amare et al., 1993); Ellenberger, et al., in preparation). The structure determined for Max homodimers does not suggest an obvious explanation for how HLH dimerization specificities might be determined, but by comparing it with other bHLH structures, it should be possible to formulate testable hypotheses.

For example, the bHLH protein E47 forms dimers with relatively high affinity (Sun and Baltimore, 1991). The E47-DNA complex structure which was determined by X-ray crystallography (Ellenberger et al., 1994) has revealed that, relative to Max,

E47 is characterized by an additional interaction between charged residues across dimer subunits. This interaction appears to occur between a histidine (H) residue at the end of helix 1 and a glutamic acid (E) residue near the end of helix 2 on the opposite subunit. It involves an increase in the length of helix 1, and appears to be potentially able to contribute significantly to dimerization (Ellenberger et al., 1994). The structures suggest that this interaction is possible because E47 lacks a particular tyrosine (Y) residue which is present within helix 2 in many bHLH proteins, including bHLH-ZIP proteins (Figure 1), and which seems to present steric constraints that prevent helix 1 from extending as far as in E47.

In collaboration with Dr. Ellenberger, we have begun to test whether this interaction is critical, by substituting the apparently relevant residues from E2A proteins into MyoD, which forms homodimers poorly (Sun and Baltimore, 1991). During the first project year, we created a series of E2A/MyoD swap mutants, of which MD/E/YQH2VE (Figure 3) contained the most E2A residues, and would have been predicted by modeling to undergo the interaction described above. Surprisingly, this protein bound DNA at a level slightly lower than wild type. During the past year we created the mutant MD/E2/YQH2VE (Figure 3), in which the entire E47 loop region was substituted into MyoD. This protein also formed dimers with an affinity that was only approximately the same as wild type. These results indicated that these substitutions did not allow the interaction described above to take place, or perhaps that the binding energy derived from it was overcome by negative effects associated with combining these particular MyoD and E2A residues.

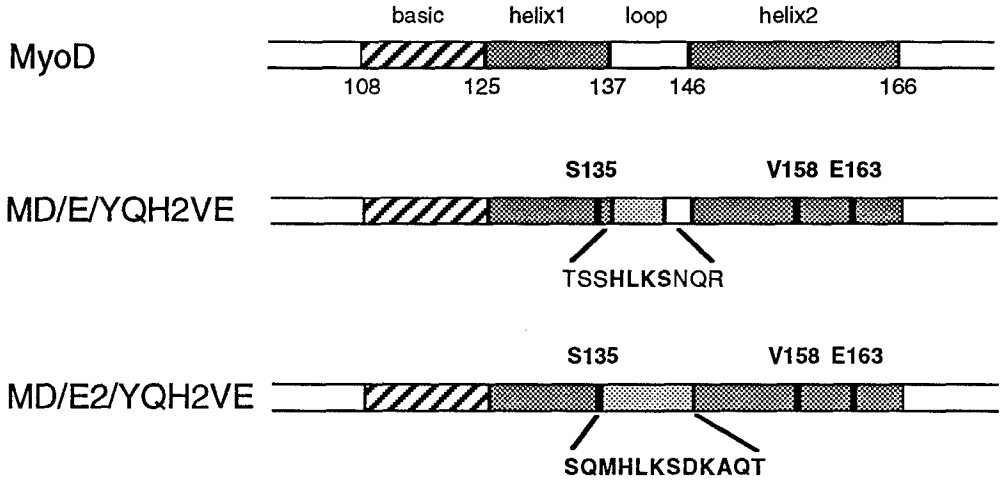


Figure 3:
MyoD HLH mutations. The basic region, helices 1 and 2, and the loop region are indicated by differently shaded boxes. Site-directed substitution mutants are indicated by the standard one-letter amino acid code. Numbering is according to the sequence of full-length MyoD. Substituted residues are indicated in bold type.

2. Protein-DNA interactions:

Fortunately, our investigations of bHLH protein-DNA interactions have met with more success. We have begun by looking at the bHLH protein MyoD, the functional capabilities of which have been studied most extensively (Davis and Weintraub, 1992). We are investigating how certain residues affect binding preferences at positions internal to and flanking the CA -- TG consensus, in particular those implicated in "positive control." We are analyzing these mutants by the selection and and amplification of binding sites (SAAB) technique of in vitro nucleic acid selection,

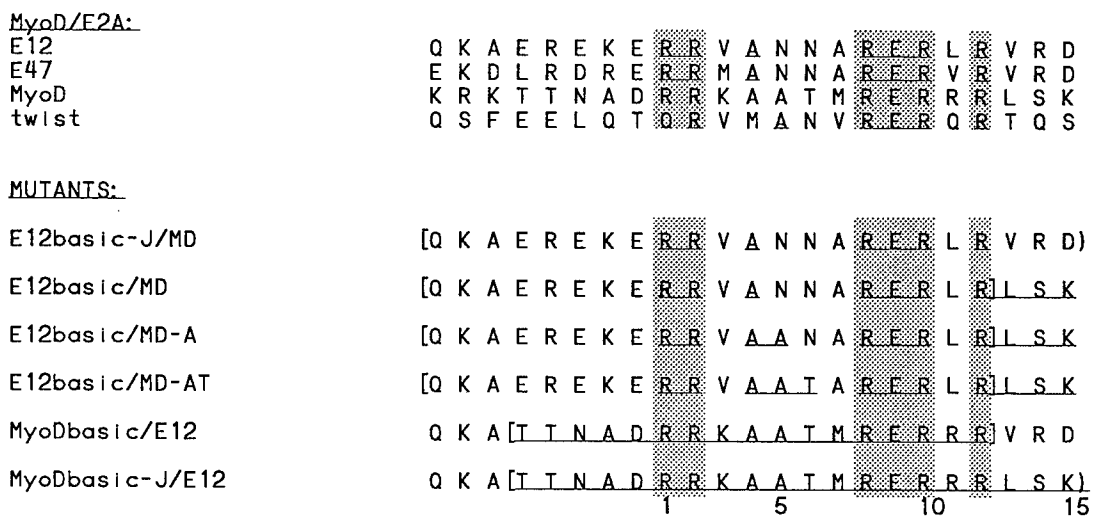
coupled with a pooled sequencing assay (Blackwell and Weintraub, 1990). In a relatively rapid fashion, we can thus assay the consequences of mutations on DNA binding, both by selecting preferred sequences and by selecting for pools of sequences to which these complexes will not bind. We are then confirming the results of these SAAB assays by analyzing binding of mutants to individual oligonucleotides. These results are expected to lead to modeling studies, which will be employed to design future mutagenesis efforts that, in the case of particularly definitive mutants, can be subjected to crystallographic analysis by Dr. Ellenberger.

Various tissue-specific bHLH proteins, such as MyoD, function as heterodimers with E2A proteins (Murre et al., 1989). These different heterodimer combinations can then bind to different versions of the CA -- TG consensus; for example, MyoD/E2A proteins bind to sites with a CACCTG core (Blackwell and Weintraub, 1990), and our experiments have now determined that heterodimers of MyoD with the bHLH protein Twist, which is involved in mesoderm specification (see (Michelson, 1996)) bind to CATATG sites (Figure 4). The observation that this preference is different over the entire site, and not over just one half, suggests that different E2A partners might recognize different sequences by positioning both bound basic regions differently on the DNA. Substitution of the BR from E12 (an E2A protein, Figure 1) for that of MyoD (E12basic/MyoD; Figure 4) results in a protein that will bind DNA with close to wild type affinity as a heterodimer with E2A, but will not induce myogenesis (Davis et al., 1990; Davis and Weintraub, 1992). Remarkably, a homodimer of this protein binds preferentially to a CATATG site that is identical to the preferential E2A/Twist (or Twist/Twist) recognition site (Figure 4). Back-substitution of the MyoD A_5 and T_6 residues into E12basic/MyoD restores the ability to induce myogenesis (Davis et al., 1990; Davis and Weintraub, 1992), and we have now shown that a homodimer of this back-substituted protein preferentially recognizes a MyoD consensus (Figure 4). This last result is striking in that "positive control" mutations have been identified at these residues. Our results suggest that, although genetic evidence indicates that these residues affect protein-protein interactions, they influence protein-DNA contact throughout the site. Indeed, in the MyoD structure obtained by Xray crystallography, these residues are oriented so that they point directly into the major groove (Ma et al., 1994). The most straightforward interpretation of our results is that these residues are involved in basic region positioning, which is in turn involved in positive control. In this interpretation, recognition of the same site by E12basic/MyoD and Twist is not a coincidence, but arises from analogous positioning of critical residues.

An additional MyoD residue of biological importance is K_{15} which, when substituted into E12 along with A_5 and T_6, confers the ability to induce myogenesis (Davis and Weintraub, 1992). This K residue is located within the BR-helix 1 junction (Figures 1 and 4). Our experiments indicate that a heterodimer of E12basic/MyoD with MyoDbasic/E12 binds preferentially to the "Twist" CATATG site (Figure 4), again indicating mis-positioning of the basic regions. This mis-positioning appears to be corrected when both corresponding junctions are also substituted (E12basic-J/MD and MDbasic-J/E12; Figure 4). These findings indicate a pivotal role for the junctions in positioning the BRs and suggest, provocatively, that the critical K residue might be involved. They are also consistent with the notion that the protein-protein interactions that are implied to involve the MyoD BR might depend on proper positioning of these BRs in the major groove, not on protein-protein interactions involving the "positive control" residues directly.

We have now begun to test this hypothesis by looking at DNA binding by additional mutant versions of MyoD. Substitution of multiple different amino acids into the A_5 and T_6 positions results in molecules that lose discrimination among CA - -TG

A. BASIC REGIONS:



B. BINDING PREFERENCES:

MyoD	GACAGCTGTC A T	Twist/E12	NCCATATGGN
E2A	NICAGGTGAN CC	Twist	NCCATATGGN
MyoD/E2A	GACAGGTGAN A CC	E12basic/MD	NCCATATGGN
E12basic/MD +MDbasic/E12	NCCATATGGN	E12basic/MD-A	NCCATATGGN
E12basic-J/MD +MDbasic-J/E12	GACAGGTGAN A CC	E12basic/MD-AT	GACAGCTGTC A T

Figure 4:

DNA binding site preferences of the indicated bHLH proteins. In A, residues conserved among all bHLH proteins are shaded. Numbering is as in the text. Brackets indicate residues that were substituted from the indicated proteins. Amino acids that are shared with MyoD are underlined in the other bHLH proteins. In B, the CANNTG consensus is indicated in bold type. Bases that are selected against are indicated by underlining.

sites (not shown). Consequently, we are taking the approach of mutating non-essential BR amino acids to alanine (Fisher et al., 1993), then swapping in combinations of critical residues from MyoD, E2A, Twist, and other bHLH proteins. By this approach, we will determine whether the "positioning" effects we have observed above can be pinpointed to those residues. These mutants are under construction by a research technician, Thip Kophengnavong. We expect that these experiments can be extended to include molecular modeling, and to address recognition of "non-canonical" sites by Myc-family bHLH proteins (Blackwell et al., 1993).

We have also attempted to identify a binding site for a novel Max dimerization partner, p18 (R. Eisenman, unpublished). This bHLH-ZIP protein dimerizes well with Max, but is not a member of either the Myc or Mad families. Heterodimers of Max and p18 do not bind well to CACGTG sites, suggesting that they may have a novel binding specificity. So far, we have not been successful in identifying a p18 recognition site.

Through a collaboration, we also developed a system for in vivo selection of regulatory sequences that respond to a given transcription factor (Huang et al.,). Sequences that allow transcriptional activation by MyoD were selected from a random sequence library, which had been cloned into a promoter in place of a required MyoD binding site. This promoter library was placed upstream of a β-gal reporter, allowing FACS selection of cells that harbored active plasmids. Three rounds of selection were performed, each of which involved co-transfection of the library DNA with a MyoD expression vector, followed by FACS selection of cells that received an "active" MyoD-responsive construct, then expansion of the selected DNA in E. coli. Remarkably, the selected functional sequences represented only a subset of the allowed MyoD binding sites, and in this system the "best" MyoD/E2A binding sites were inactive. These findings suggest that either binding in an appropriate conformation, or binding to a particular sequence, may be required for transcriptional activation by MyoD. These results are of particular interest in light of the importance of BR positioning that is implied by the experiments described above. It will be of significant interest to investigate the basis for this finding, and to determine whether such mechanisms might be characteristic of other bHLH proteins.

B. Investigation of DNA binding by the Mastermind protein:

In our second specific aim, we had originally proposed to use *in vitro* selection to isolate single stranded nucleic acid molecules (aptamers) that could bind to c-Myc and inhibit its dimerization or DNA binding (Ellington and Szostak, 1990). Since the original proposal was submitted, it has become apparent that this technology is being pursued vigorously by numerous biotechnology companies, many of which have chemistry departments that can readily synthesize a variety of modified nucleotides that can be used in these experiments. In light of the number and breadth of those efforts, I have chosen to devote my Army Breast Cancer Program award strictly to basic research, and have begun to investigate DNA binding by the Mastermind protein. It is hoped that this research will provide novel insights into Notch function, and thus into a pathway that is linked to mammary carcinoma.

Ms Kophengnavong has begun performing *in vitro* selections for Mam binding sites, using a GST fusion protein that contains a fragment of Mam that includes its BR. So far, we have determined that this Mam fragment binds DNA non-specifically at an affinity of approximately 100 nM, an observation which is a promising indicator that it may function as a DNA binding protein. We are continuing our selections to search for a specific Mam binding site.

CONCLUSIONS:

Our investigations of bHLH protein dimerization are as yet incomplete, because the mutagenesis performed so far has yielded inconclusive results. However, the structural evidence for the model under investigation is compelling. Bearing this in mind, we will continue to pursue our test of this hypothesis by substituting successively larger regions of E47 into MyoD. However, we have prioritized our DNA-binding experiments, which have met with considerably more success.

Our studies of bHLH protein-DNA binding indicate the novel finding that BR positioning may underlie recognition of different CA -- TG sites by different dimers of E2A and its partners, an issue that has till now remained a mystery. More importantly, this phenomenon may be linked to the biological activity of these proteins. For now we will concentrate on MyoD, the biological roles of which have been more widely studied than those of the Myc proteins, and on other E2A partners. We will next expand these experiments to address how Myc proteins recognize the non-canonical sequences.

The system that we have developed for in vivo selection of functional binding sites (Huang et al., 1996) will prove useful in a variety of areas, because it allows identification and comparison of regulatory sites that respond to a particular protein in the context of different promoters and co-factors. It may ultimately prove helpful for investigations of how Myc can activate some genes and repress others.

As indicated above, our finding that Mam binds to DNA at least non-specifically is encouraging, and is consistent with the idea that it functions as a DNA binding protein. These experiments, if successful, will fill in an important missing link in the Notch pathway, which has been linked to mammary carcinoma. They will also open the possibility of using biochemical strategies to isolate vertebrate Mam homologs.

REFERENCES:

Alex, R., Sozeri, O., Meyer, S., and Dildrop, R. (1992). Determination of the DNA sequence recognized by the bHLH-zip domain of the N-Myc protein. Nucleic Acids Research *20*, 2257-2263.

Amati, B., Brooks, M. W., Levy, N., Littlewood, T. D., Evan, G., and Land, H. (1993). Oncogenic activity of the c-Myc protein requires dimerization with Max. Cell *72*, 233-245.

Amati, B., Dalton, S., Brooks, M. W., Littlewood, T. D., Evan, G. I., and Land, H. (1992). Transcriptional activation by the c-Myc oncoprotein in yeast requires interaction with Max. Nature *359*, 423-426.

Amin, C., Wagner, A. J., and Hay, N. (1993). Sequence-specific transcriptional activation by Myc and repression by Max. Mol. Cell. Biol. *13*, 383-390.

Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M. E. (1995). Notch signaling. Science *268*, 225-232.

Ayer, D., Kretzner, L., and Eisenman, R. (1993). Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell *72*, 211-222.

Beckmann, H., and Kadesch, T. (1991). The leucine zipper of TFE3 dictates helix-loop-helix dimerization specificity. Genes & Development *5*, 1057-1066.

Beckmann, H., Su, L. K., and Kadesch, T. (1990). TFE3: a helix-loop-helix protein that activates transcription through the immunoglobulin enhancer μE3 motif. Genes & Development *4*, 167-179.

Bello-Fernandez, C., Packham, G., and Cleveland, J. L. (1993). The ornithine decarboxylase gene is a transcriptional target of c-Myc. Proc Natl Acad Sci U S A *90*, 7804-7808.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell *61*, 49-59.

Benvenisty, N., Leder, A., Kuo, A., and Leder, P. (1992). An embryonically expressed gene is a target for c-Myc regulation via the c-Myc binding sequence. Genes & Development *6*, 2513-2523.

Berberich, S., Hyde, D. N., Espenshade, P., and Cole, M. (1992). max encodes a sequence-specific DNA-binding protein and is not regulated by serum growth factors. Oncogene *7*, 775-779.

Blackwell, T. K. (1995). Selection of protein binding sites from random nucleic acid sequences. Methods in Enzymology *254*, 604-618.

Blackwell, T. K., Bowerman, B., Priess, J., and Weintraub, H. (1994). Formation of a monomeric DNA binding domain by Skn-1 bZIP and homeodomain elements. Science *266*, 621-628.

Blackwell, T. K., Huang, J., Ma, A., Kretzner, L., Alt, F. W., Eisenman, R. N., and Weintraub, H. (1993). Binding of Myc-family proteins to canonical and non-canonical DNA sequences. Molecular and Cellular Biology *13*, 5216-5224.

Blackwell, T. K., Kretzner, L., Blackwood, E. M., Eisenman, R. N., and Weintraub, H. (1990). Sequence-specific DNA binding by the c-Myc protein. Science *250*, 1149-1151.

Blackwell, T. K., and Weintraub, H. (1990). Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science *250*, 1104-1110.

Blackwood, E. M., and Eisenman, R. N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science *251*, 1211-1217.

Blackwood, E. M., Luscher, B., and Eisenman, R. N. (1992). Myc and Max associate in vivo. Genes & Development *6*, 71-80.

Born, T. L., Frost, J. A., Schönthal, A., Prendergast, G. C., and Feramisco, J. R. (1994). C-Myc cooperates with activated Ras to induce the *cdc2* promoter. Mol Cell Biol *14*, 5710-5718.

Bowerman, B., Eaton, B. A., and Priess, J. R. (1992). *skn-1*, a maternally expressed gene required to specify the fate of ventral blastomeres in the early *C. elegans* embryo. Cell *68*, 1061-1075.

Carr, C. S., and Sharp, P. A. (1990). A helix-loop-helix protein related to the immunoglobulin E box-binding proteins. Molecular & Cellular Biology *10*, 4384-4388.

Charron, J., Malynn, B. A., Fisher, P., Stewart, V., Jeannotte, L., Goff, S. P., Robertson, E. J., and Alt, F. W. (1992). Embryonic lethality in mice homozygous for a targeted disruption of the N-*myc* gene. Genes & Development *6*, 2248-2257.

Collum, R. G., and Alt, F. W. (1990). Are myc proteins transcription factors? Cancer Cells *2*, 69-75.

Daksis, J. I., Lu, R. Y., Facchini, L. M., Marhin, W. W., and Penn, L. J. Z. (1994). Myc induces cyclin D1 expression in the absence of *de novo* protein synthesis and links mitogen-stimulated signal transduction to the cell cycle. Oncogene *9*, 3635-3645.

Dang, C. V., Dolde, C., Gillison, M. L., and Kato, G. J. (1992). Discrimination between related DNA sites by a single amino acid residue of Myc-related basic-helix-loop-helix proteins. Proceedings of the National Academy of Sciences of the United States of America *89*, 599-602.

Davis, A. C., Wims, M., Spotts, G. D., Hann, S. R., and Bradley, A. (1993). A null c-*myc* mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. Genes & Development *7*, 671-682.

Davis, L., and Halazonetis, T. (1993). Both the helix-loop-helix and the leucine zipper motifs of c-Myc contribute to its dimerization specificity with Max. Oncogene *8*, 125-132.

Davis, R. L., Cheng, P. F., Lassar, A. B., and Weintraub, H. (1990). The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell *60*, 733-746.

Davis, R. L., and Weintraub, H. (1992). Acquisition of myogenic specificity by replacement of three amino acid residues from MyoD into E12. Science *256*, 1027-30.

Davis, R. L., Weintraub, H., and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell *51*, 987-1000.

Eilers, M., Schirm, S., and Bishop, J. M. (1991). The MYC protein activates transcription of the alpha-prothymosin gene. Embo Journal *10*, 133-141.

Ellenberger, T., Fass, D., Arnaud, M., and Harrison, S. C. (1994). Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. Genes & Development *8*, 970-980.

Ellenberger, T. E., Brandl, C. J., Struhl, K., and Harrison, S. C. (1992). The GCN4 basic region-leucine zipper binds DNA as a dimer of uninterrupted α-helices: Crystal structure of the protein-DNA complex. Cell *71*, 1223-1237.

Ellington, A. D., and Szostak, J. W. (1990). In vitro selection of RNA molecules that bind specific ligands. Nature *346*, 818-822.

Escot, C., Theilet, C., Lidereau, R., Spyvatos, F., Champeme, M. H., Gest, J., and Callahan, R. (1986). Genetic alteration of the c-Myc protooncogene (MYC) in human primary breast carcinomas. Proc. Natl. Acad. Sci. USA *83*, 4834-4838.

Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., and Hancock, D. C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. Cell *69*, 119-128.

Ferre-D' Amare, A. R., Prendergast, G. C., Ziff, E. B., and Burley, S. K. (1993). Recognition by Max of its cognate DNA through a dimeric b/HLH/Z domain. Nature *363*, 38-45.

Fisher, D. E., Carr, C. S., Parent, L. A., and Sharp, P. A. (1991). TFEB has DNA-binding and oligomerization properties of a unique helix-loop-helix/leucine-zipper family. Genes & Development *5*, 2342-2352.

Fisher, D. E., Parent, L. A., and Sharp, P. A. (1993). High Affinity DNA-Binding Myc Analogs: Recognition by an α Helix. Cell *72*, 467-476.

Fisher, F., and Goding, C. R. (1992). Single amino acid substitutions alter helix-loop-helix protein specificity for bases flanking the core CANNTG motif. EMBO Journal *11*, 4103-4109.

Fortini, M. E., and Artavanis-Tsakonas, S. (1994). The suppressor of hairless protein participates in Notch receptor signaling. Cell *79*, 273-282.

Galaktionov, K., Chen, X., and Beach, D. (1996). Cdc25 cell-cycle phosphatase as a target of c-*myc*. Nature *382*, 511-517.

Girard, L., Hanna, Z., Beaulieu, N., Hoemann, C. D., Simard, C., Kozak, C. A., and Jolicoeur, P. (1996). Frequent provirus insertional mutagenesis of *Notch1* in thymomas of MMTV^{D}/myc transgenic mice suggests a collaboration of c-*myc* and *Notch1* for oncogenesis. Genes Dev. *10*, 1930-1944.

Grandori, C., Mac, J., Siëbelt, F., Ayer, D. E., and Eisenman, R. N. (1996). Myc-Max heterodimers activate a DEAD box gene and interact with multiple E box-related sites in vivo. EMBO J. *15*, 4344-4357.

Gregor, P. D., Sawadogo, M., and Roeder, R. G. (1990). The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. Genes & Development *4*, 1730-1740.

Gu, W., Cechova, K., Tassi, V., and Dalla-Favera, R. (1993). Opposite regulation of gene transcription and cell proliferation by c-Myc and Max. Proc. Natl. Acad. Sci. USA *90*, 2935-2939.

Halazonetis, T. D., and Kandil, A. N. (1991). Determination of the c-MYC DNA-binding site. Proceedings of the National Academy of Sciences of the United States of America *88*, 6162-6166.

Halazonetis, T. D., and Kandil, A. N. (1992). Predicted structural similarities of the DNA binding domains of c-Myc and endonuclease Eco RI. Science *255*, 464-466.

Hann, S. R., Dixit, M., Sears, R. C., and Sealy, L. (1994). The alternatively initiated c-Myc proteins differentially regulate transcription through a noncanonical DNA-binding site. Genes & Development *8*, 2441-2452.

Harris, J. R., Lippman, M. E., Veronesi, U., and Willett, W. (1992). Breast cancer (third of three parts). New Eng. J. Med. *327*, 473-480.

Hochschild, A., Irwin, N., and Ptashne, M. (1983). Repressor structure and the mechanism of positive control. Cell *32*, 319-325.

Huang, J., Blackwell, T. K., Kedes, L., and Weintraub, H. (1996). Differences between MyoD DNA binding and activation site requirements revealed by a functional random sequence selection. Mol. Cell Biol. *16*, 3893-3900.

Jansen-Durr, P., Meichle, A., Steiner, P., Pagano, M., Finke, K., Botz, J., Wessbecher, J., Draetta, G., and Eilers, M. (1993). Differential modulation of cyclin gene expression by Myc. Proc. Natl. Acad. Sci. USA *90*, 3685-3689.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R., and Israel, A. (1995). Signalling downstream of activated mammalian Notch. Nature *377*, 355-358.

Jhappan, C., Gallahan, D., Stahle, C., Chu, E., Smith, G. H., Merlino, G., and Callahan, R. (1992). Expression of an activated *Notch*-related *int*-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. Genes Dev. *6*, 345-355.

Johnson, P. F., and McKnight, S. L. (1989). Eukaryotic transcriptional regulatory proteins. Annual Review of Biochemistry *58*, 799-839.

Jones, R. M., Branda, J., Johnston, K. A., Polymenis, M., Gadd, M., Rustgi, A., Callanan, L., and Schmidt, E. V. (1996). An essential E box in the promoter of the gene encoding the mRNA cap-binding protein (eukaryotic initiation factor 4E) is a target for activation by c-myc. Mol. Cell. Biol. *16*, 4754-4764.

Kato, G. J., Barrett, J., Villa, G. M., and Dang, C. V. (1990). An amino-terminal c-myc domain required for neoplastic transformation activates transcription. Molecular & Cellular Biology *10*, 5914-5920.

Kato, G. J., Lee, W. M., Chen, L. L., and Dang, C. V. (1992). Max: functional domains and interaction with c-Myc. Genes & Development *6*, 81-92.

Kerkhoff, E., Bister, K., and Klempnauer, K.-H. (1991). Sequence-specific DNA binding by Myc proteins. Proceedings of the National Academy of Sciences of the United States of America *88*, 4323-4327.

Kretzner, L., Blackwood, E. M., and Eisenman, R. N. (1992). Myc and Max proteins possess distinct transcriptional activities. Nature *359*, 426-429.

Kristie, T. M., and Sharp, P. A. (1990). Interactions of the Oct-1 POU subdomains with specific DNA sequences and with the HSV alpha-trans-activator protein. Genes & Development *4*, 2383-96.

Lai, J.-S., Cleary, M. A., and Herr, W. (1992). A single amino acid exchange transfers VP-16-induced positive control from the Oct-1 to the Oct-2 homeodomain. Genes & Development *11*, 2058-2065.

Landschulz, W. H., Johnson, P. F., and McKnight, S. L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science *240*, 1759-1764.

Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., Hauschka, S. D., and Weintraub, H. (1989). MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. Cell *58*, 823-831.

Lassar, A. B., Davis, R. L., Wright, W. E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D., and Weintraub, H. (1991). Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. Cell *66*, 305-15.

Leder, A., Pattengale, P. K., Kuo, A., Stewart, T. A., and Leder, P. (1986). Consequences of widespread deregulation of the c-*myc* gene in transgenic mice: multiple neoplasms and normal development. Cell *45*, 485-495.

Lu, X., Park, S. H., Thompson, T. C., and Lane, D. P. (1992). *ras*-induced hyperplasia occurs with mutation of p53, but activated *ras* and *myc* together can induce carcinoma without p53 mutation. Cell *70*, 153-161.

Luscher, B., and Eisenman, R. N. (1990). New light on Myc and Myb. Part I. Myc. Genes & Development *4*, 2025-2035.

Ma, A., Moroy, T., Collum, R., Weintraub, H., Alt, F. W., and Blackwell, T. K. (1993). DNA-binding by N- and L-Myc proteins. Oncogene *8*, 1093-1098.

Ma, P. C., Rould, M. A., Weintraub, H., and Pabo, C. O. (1994). Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. Cell *77*, 451-459.

Michelson, A. (1996). A new turn (or two) for Twist. Science *272*, 1449-1450.

Moens, C. B., Auerbach, A. B., Conlon, R. A., Joyner, A. L., and Rossant, J. (1992). A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung. Genes & Development *6*, 691-704.

Mukherjee, B., Morgenbesser, S. D., and DePinho, R. A. (1992). Myc family oncoproteins function through a common pathway to transform normal cells in culture: cross-interference by Max and trans-acting dominant mutants. Genes & Development *6*, 1480-1492.

Murre, C., McCaw, P. S., and Baltimore, D. (1989). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell *56*, 777-783.

Murre, C., McCaw, P. S., Vaessin, H., Caudy, M., Jan, L. Y., Jan, Y. N., Cabrera, C. V., Buskin, J. N., Hauschka, S. D., Lassar, A. B., and et, a. l. (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell *58*, 537-544.

Neiman, P. E., Thomas, S. J., and Loring, G. (1991). Induction of apoptosis during normal and neoplastic B-cell development in the bursa of Fabricius. Proceedings of the National Academy of Sciences of the United States of America *88*, 5857-5861.

O'Shea, E. K., Rutkowski, R., and Kim, P. S. (1989). Evidence that the leucine zipper is a coiled coil. Science *243*, 538-542.

O'Shea, E. K., Rutkowski, R., and Kim, P. S. (1992). Mechanism of specificity in the Fos-Jun oncoprotein heterodimer. Cell *68*, 699-708.

Papoulas, O., Williams, N., and Kingston, R. E. (1992). DNA binding activities of c-Myc purified from eukaryotic cells. The Journal of Biological Chemistry *267*, 10470-10480.

Perutz, M. (1992). Protein structure: New approaches to disease and therapy (New York: W. H. Freeman and Company).

Prendergast, G. C., Hopewell, R., Gorham, B. J., and Ziff, E. B. (1992). Biphasic effect of Max on Myc cotransformation activity and dependence on amino- and carboxy-terminal Max functions. Genes & Development *6*, 2429-2439.

Prendergast, G. C., Lawe, D., and Ziff, E. B. (1991). Association of Myn, the murine homolog of max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. Cell *65*, 395-407.

Prochownik, E. V., and Van Antwerp, M. E. (1993). Differential patterns of DNA-binding by myc and max proteins. Proc. Natl. Acad. Sci. USA *90*, 960-964.

Reddy, C. D., Dasgupta, P., Saikumar, P., Dudek, H., Rauscher, F. J., and Reddy, E. P. (1992). Mutational role of Max: role of basic, helix-loop-helix/leucine zipper domains in DNA-binding, dimerization, and regulation of Myc-mediated transcriptional activation. Oncogene *7*, 2085-2092.

Robbins, J., Blondel, B. J., Gallahan, D., and Callahan, R. (1992). Mouse mammary tumor gene *int-3* : a member of the *notch* gene family transforms mammary epithelial cells. J. Virol. *66*, 2594-2599.

Roy, A. L., Carruthers, C., Gutjahr, T., and Roeder, R. G. (1993). Direct role for Myc in transcription initiation mediated by interactions with TFII-I. Nature *365*, 359-361.

Sawai, S., Shimono, A., Wakamatsu, Y., Palmes, C., Hanaoka, K., and Kondoh, H. (1993). Defects on embryonic organogenesis resulting from targeted disruption of the N-*myc* gene in the mouse. Development *117*, 1445-1455.

Sawyers, C. L., Callahan, W., and Witte, O. N. (1992). Dominant negative Myc blocks transformation by Abl oncogenes. Cell *70*, 901-910.

Schoenenberger, C.-A., Andres, A.-C., Groner, B., van der Valk, M., LeMeur, M., and Gerlinger, P. (1988). Targeted c-*myc* gene expression in mammary glands of transgenic mice induces mammary tumours with constitutive milk protein gene transcription. EMBO Journal *7*, 169-175.

Schreiber-Agus, N., Horner, J., Torres, R., Chiu, F.-C., and DePinho, R. (1993). Zebra fish *myc* family and *max* genes: differential expression and oncogenic activity throughout vertebrate evolution. Mol. Cell. Biol. *13*, 2765-2775.

Schwarz, J. J., Chakraborty, T., Martin, J., Zhou, J. M., and Olson, E. N. (1992). The basic region of myogenin cooperates with two transcription activation domains to induce muscle-specific transcription. Mol. Cell. Biol. *12*, 266-275.

Shi, Y., Glynn, J. M., Guilbert, L. J., Cotter, T. G., Bissonette, R. P., and Green, D. (1992). Role for c-Myc in activation-induced apoptotic cell death in T-cell hybridomas. Science *257*, 212-214.

Smith, G. H., Gallahan, D., Diella, F., Jhappan, C., Merlino, G., and Callahan, R. (1995). Constitutive expression of a truncated *INT3* gene in mouse mammary epithelium impairs differentiation and functional development. Cell Growth Differ. *6*, 563-577.

Smoller, D., Friedel, C., Schmid, A., Bettler, D., Lam, L., and Yedvobnick, B. (1990). The *Drosophila* neurogenic locus *mastermind* encodes a nuclear protein unusually rich in amino acid homopolymers. Genes Dev. *4*, 1688-1700.

Stanton, B. R., Perkins, A. S., Tessarollo, L., Sassoon, D. A., and Parada, L. F. (1992). Loss of N-*myc* function results in embryonic lethality and failure of the epithelial component of the embryo to develop. Genes & Development *6*, 2235-2247.

Stern, S., Tanaka, M., and Herr, W. (1989). The Oct-1 homeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. Nature *341*, 624-630.

Stewart, T. A., Pattengale, P. K., and Leder, P. (1984). Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell *38*, 627-637.

Stone, J., de Lange, T., Ramsay, G., Jakobovits, E., Bishop, J. M., Varmus, H., and Lee, W. (1987). Definition of regions in human c-Myc that are involved in transformation and nuclear localization. Mol. Cell. Biol. *7*, 1697-1709.

Sun, X. H., and Baltimore, D. (1991). An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. Cell *64*, 459-470.

Van Antwerp, M. E., Chen, D. G., Chang, C., and Prochownik, E. V. (1992). A point mutation in the MyoD basic domain imparts c-Myc-like properties. Proceedings of the National Academy of Sciences of the United States of America *89*, 9010-9014.

van Leeuwen, F., and Nusse, R. (1995). Oncogene activation and oncogene cooperation in MMTV-induced mouse mammary cancer. Semin. Cancer Biol. *6*, 127-133.

Vinson, C. R., Hai, T., and Boyd, S. M. (1993). Dimerization specificity of the leucine zipper-containing bZIP motif on DNA-binding: prediction and rational design. Genes & Development *7*, 1047-1058.

Voronova, A., and Baltimore, D. (1990). Mutations that disrupt DNA binding and dimer formation in the E47 helix-loop-helix protein map to distinct domains. Proceedings of the National Academy of Sciences of the United States of America *87*, 4722-4726.

Walker, C. W., Boom, J. D. G., and Marsh, A. (1992). First non-vertebrate member of the myc gene family is seasonally expressed in an invertebrate testis. Oncogene *7*, 2007-2012.

Watson, P. H., Safneck, J. R., Le, K., Dubik, D., and Shiu, R. P. C. (1993). Relationship of c-myc amplification to progression of breast cancer from in situ to invasive tumor and lymph node metastasis. J. Natl. Cancer Inst. *85*, 902-907.

Weintraub, H., Davis, R., Tapscott, S., Thayer, M., Krause, M., Benezra, R., Blackwell, T. K., Turner, D., Rupp, R., Hollenberg, S., Zhuang, Y., and Lassar, A. (1991). The MyoD gene family: nodal point during specification of the muscle cell lineage. Science *251*, 761-766.

Weintraub, H., Dwarki, V. J., Verma, I., Davis, R., Hollenberg, S., Snider, L., Lassar, A., and Tapscott, S. J. (1991). Muscle-specific transcriptional activation by MyoD. Genes & Development *5*, 1377-86.

Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., Lassar, A. B., and Miller, A. D. (1989). Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proceedings of the National Academy of Sciences of the United States of America *86*, 5434-8.

Wenzel, A., Cziepluch, C., Hamann, U., Schurmann, J., and Schwab, M. (1991). The N-Myc oncoprotein is associated in vivo with the phosphoprotein Max(p20/22) in human neuroblastoma cells. Embo Journal *10*, 3703-3712.

Zervos, A. S., Gyuris, J., and Brent, R. (1993). Mxi-1, a Protein That Specifically Interacts with Max to bind Myc-Max Recognition Sites. Cell *72*, 223-232.